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Molecular Pathways Needed for Regeneration of Spinal Cord and Muscle in a Vertebrate

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Summary

The tail of the frog tadpole, comprising spinal cord, muscle, and notochord, regenerates following partial amputation. We show that, in *Xenopus*, this occurs throughout development, except for a "refractory period" between stages 45 and 47, when tails heal over without regeneration. Regeneration can be enabled during this refractory period by activation of either the BMP or Notch signaling pathways. Conversely, regeneration can be prevented during the later, regenerative, stages by inhibition of either pathway. BMP signaling will cause regeneration of all tissues, whereas Notch signaling activates regeneration of spinal cord and notochord, but not muscle. An activated form of Msx1 can promote regeneration in the same way as BMP signaling. Epistasis experiments suggest that BMP signaling is upstream of Notch signaling but exerts an independent effect on muscle regeneration. The results demonstrate that regenerative capability can be enabled by genetic modifications that reactivate specific components of the developmental program.

Introduction

Epimorphic regeneration is defined as the regrowth of amputated structures from an anatomically complex stump (Goss, 1991). While thought to be a latent property of all vertebrates, it occurs rarely in adults, and has been most extensively studied in urodele amphibians, which can regenerate limbs and tails as adults (reviewed in Brockes, 1997). Anuran tadpoles can also regenerate their tails, including spinal cord, notochord, and myotomes, up until metamorphosis (Bosco, 1979; Filoni et al., 1977). This involves mobilization of cells from the tissues of the stump to form a regenerating bud, growth of this bud, and redifferentiation of the various tissue types to produce a near perfect replacement of the original. While it is often suggested that anurans only regenerate a simple, ependymal tube, lacking nerve fibers, neurons, and ganglion cells (Goss, 1969; Piatt, 1955), in actual fact, the only defect in regenerated tails of *Xenopus, Rana*, or *Discoglossus* tadpoles is the failure of the spinal ganglia to reform (Filoni and Bosco, 1981).

Study of urodeles has provided much information about gene expression during appendage regeneration (reviewed in Gardiner et al., 1999), but the molecular biology technologies available for working on urodeles are currently limited. By contrast, in *Xenopus*, it is possible to manipulate the expression of candidate factors involved in regeneration using the transgenic system developed by Amaya and Kroll (1999). Furthermore, the temperature-sensitive heat shock promoter HSP70 (Wheeler et al., 2000) can be used to activate or inhibit selected genes at postembryonic stages. We have used these new techniques to investigate the molecular mechanisms of axial regeneration.

We show that regeneration of tails does not, as previously thought, occur at all stages of development. While the embryonic tail is able to regenerate all the axial tissues (muscle, spinal cord, and notochord), regenerative ability is consistently lost between stages 45 and 47 (4–6 days of development) and is regained after stage 48. The presence of this refractory period has allowed us to study for the first time, to our knowledge, the mechanism of acquisition of axial regenerative ability in a vertebrate. We find that the ability to regenerate a tail correlates with reexpression of genes that are involved in the BMP and Notch signaling cascades, the same pathways that drive embryonic tail development (Beck and Slack, 1999; Beck et al., 2001). Using an inducible transgenic system, we demonstrate that reactivation of these developmental pathways is necessary for regeneration. Furthermore, activation of these pathways during the refractory stage is sufficient to drive regeneration, resulting in replacement of lost tissues. We have therefore begun to unravel the mechanisms that drive epimorphic regeneration in the *Xenopus* tail. Whether activation of the same pathways could be used to achieve regeneration of differentiated axial tissues in higher vertebrates will be an interesting question for the future.

Results

Analysis of Stage-Specific Regeneration Competence in *Xenopus* Tadpoles

While *Xenopus* tadpoles can regenerate their tails up to metamorphosis, the embryonic tail bud cannot regenerate (Tucker and Slack, 1995), and removal of even a small part of the tail bud causes a defect in the final tail. Because of this discordance between embryonic and tadpole behavior, we investigated the ability of tadpoles to regenerate tails during the period from 3 to 7 days old (stages 42–48). Tadpoles from 11 spawnings from females acquired from two different sources were subjected to 50% tail amputation at stages 42, 46/47, and 48. In all cases, regeneration was significantly reduced in stage 46/47 tadpoles compared to stage 42 or 48 (Figures 1A–1C). When all the data were pooled and standard errors were calculated, 68% of stage 42 tails regenerated ($\pm$6%, N = 299) compared to 7% of stage 46/47 tails ($\pm$2%, N = 392) and 67% of stage 48 tails ($\pm$8%, N = 270). Regeneration capability increases to near 100% in stage 49 and later tadpoles, and is then maintained until loss of the tail at metamorphic climax.

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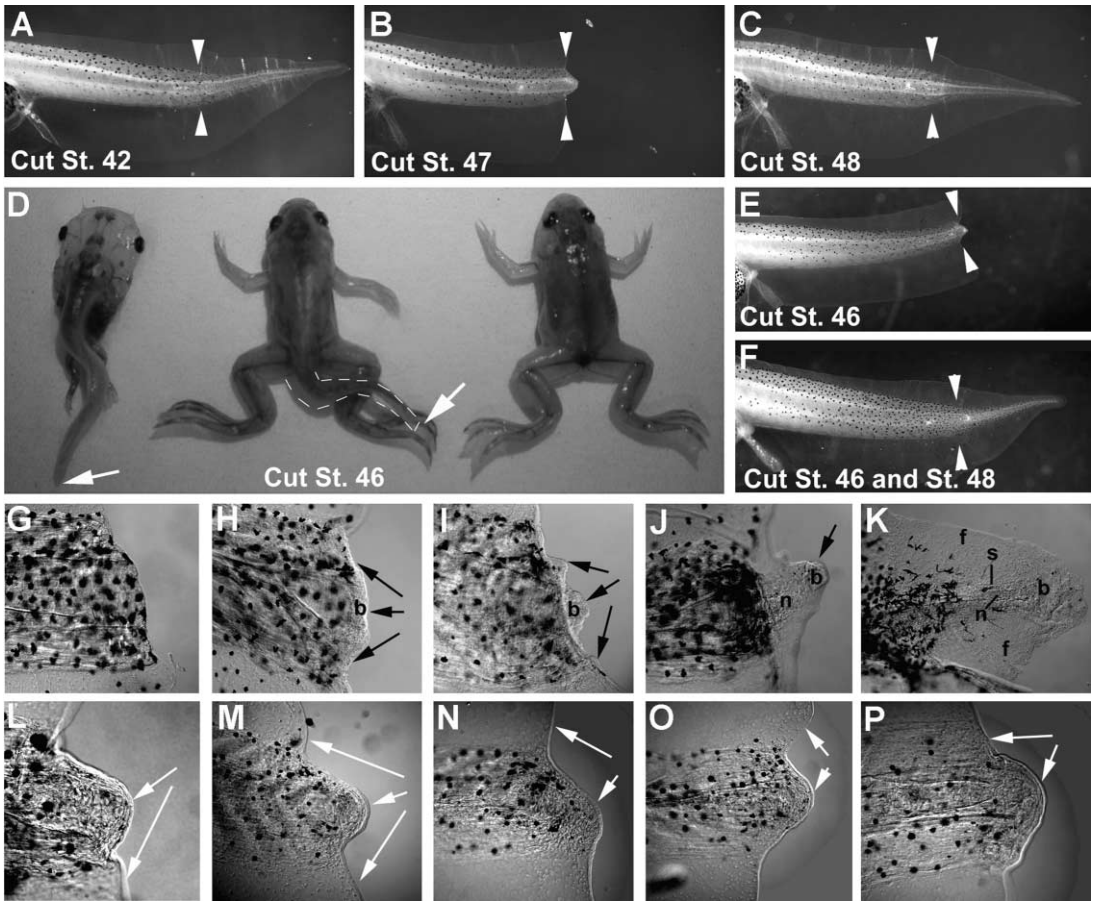

Figure 1. Axial Regenerative Ability in *Xenopus laevis* Tadpoles Is Lost at Stage 45/47 and Regained at Stage 48 and Later

White arrowheads show the level of tail amputation where appropriate.

(A–C) Tadpoles were subjected to removal of 50% of the postanal tail at different stages of development and allowed to recover for 7 days. Stage 42 (A), stage 47 (B), and stage 48 (C).

(D) Tadpoles subjected to 50% tail removal at stage 46 do not regenerate a tail, but develop normally, and the remaining stump is resorbed normally during metamorphosis. Left to right: stage 58; stage 63; stage 66.

(E and F) Tadpole tails amputated at stage 46 (E) will regenerate if they are cut again after stage 48 (F).

(G–P) Tail stumps partially cleared in glycerol and viewed under Nomarski optics.

(G–K) Regeneration process in stage 49 tadpoles. Wound epidermis is indicated by black arrows.

(G) Stage 49 tail fixed immediately after amputation.

(H) After 24 hr, the wound epithelium has formed and blastemal cells are appearing.

(I) Within 48 hr, blastemal cells have migrated into the center of the lesion and are proliferating ("cone" stage).

(J) After 3 days, the proximal blastemal cells are beginning to redifferentiate into the constituent tissues of the tail.

(K) By 4–5 days, new notochord, spinal cord, fin, and presomitic mesoderm have formed and melanocytes (black pigment cells) are migrating into the new tail.

(L–P) In contrast, tails cut at stage 47 do not regenerate or form a blastema, and the cut surface becomes covered with a skin-like epithelium (white arrows).

(L) By 24 hr postamputation, a skin-like epithelium has formed over the cut surface of the tail stump (white arrows). The tissue behind this epithelium does not form a blastema.

(M–P) The wound area does not alter over time, and there is no regeneration of tail tissues.

(M–P) After 48 hr (M), 3 days (N), 4 days (O), and 7 days (P) postamputation. All tail stumps are lateral views oriented anterior to the left, and dorsal uppermost. b, regeneration bud; f, fin; n, notochord; s, spinal cord.

When nonregenerating cases were kept, they developed normally to later stages and would even undergo metamorphosis to froglets, but did not regenerate the tail (Figures 1D and 1E) except in rare cases, possibly resulting from damage to the stump (1/11 cases). However, if the tail of such cases was reamputated just proximal to the stump after stage 48, normal regeneration did take place, resulting in the formation of a complete new tail (10/10 cases; Figure 1F). *Xenopus* tadpoles therefore exhibit stage-dependent regenerating and nonregenerating capability of tails as well as limbs (Dent, 1962; Muneoka et al., 1986; Overton, 1963). In the limbs,

regeneration ability is lost in later stages, coincident with the onset of ossification of the limb skeleton (Wolfe et al., 2000).

We used Nomarski optics to examine the regenerating and nonregenerating tail stumps over 4 days (Figures 1G–1P). In regenerating tadpole tails, a wound epithelium forms rapidly, covering the wound after 6–12 hr, and within 24 hr undifferentiated cells appear immediately beneath this epithelium (Figure 1H). In 48 hr, a cone-shaped bud forms (Figure 1I), and over the next 2 days, it expands rapidly, and the proximal tissues begin to differentiate into the spinal cord, notochord, myotomes, and fin of the regenerated tail (Figures 1J and 1K). (Detailed anatomical study of the regenerating bud will be reported separately; C. Gargioli and J.M.W.S., unpublished data). The situation in the nonregenerating tadpole tails was very different. Healing of the wound was observed to take place more slowly. By 24 hr after amputation, a thick skin-like epithelium has formed over the cut surface, and no accumulation of dedifferentiated tissue is seen (Figure 1L). These tail stumps never form a regenerating bud and there is no tissue regeneration (Figures 1M–1P). We conclude that there is a switch between two types of response to tail amputation. Amputation during the refractory period results in slow healing, with formation of a full thickness epidermis over the wound surface. No undifferentiated cells are seen to accumulate beneath this epidermis. In contrast, amputation at stage 49 results in much faster healing and formation of a specialized wound epidermis, followed by mobilization of cells from the stump, bud formation, and replacement of lost tissues.

Genes with Roles in Tail Development Are Reexpressed in Regenerating Tails

It has long been postulated that epimorphic regeneration, via the formation of a growing bud, recapitulates development (Stocum, 1984). By contrast with our relative ignorance of the mechanisms of regeneration, the genetic pathways involved in tail development are quite well understood, with the BMP and Notch pathways being required for formation of the tail bud-derived somites and neural tube, respectively (Beck and Slack, 1999, 2002; Beck et al., 2001). We have now analyzed the expression of several key tail development genes in regenerating and nonregenerating tail stumps (Figure 2). We find that genes expressed in the tail bud during early developmental stages are almost always reexpressed in regenerating tails, but not in nonregenerating tails. The reexpression of developmental genes is not an immediate-early response to wounding, and seems to require prior formation of a regeneration bud. Genes involved in the Notch (*Notch-1*, *X-delta-1*, and *Lfng*) and BMP (*BMP4, Msx 1, and 2*) signaling pathways are all reexpressed in the regeneration bud, within 24 hr of tail amputation, as is the T box transcription factor *Xbra*. All the genes shown except *Lfng*, which is upregulated from a basal low level during regeneration, have expression specific to the regeneration bud, and are not normally expressed in the tail at the level of amputation, nor are they expressed in the nonregenerating tails amputated during the refractory period.

Activating BMP or Notch Signaling Stimulates Nonregenerating Tails to Regenerate

To investigate whether reactivation of developmental pathways is sufficient to stimulate the regeneration program, we used a transgenic approach. Because interference with developmental pathways active during embryogenesis is likely to prevent generation of viable tadpoles for study, we utilized the normally silent *Xenopus* heat shock promoter HSP70 (Bienz, 1984) previously used by others (Marsh-Armstrong et al., 1999; Wheeler et al., 2000) as an inducible transgene system in *Xenopus*. We have further validated this system by creating transgenic tadpoles in which the HSP70 promoter drives either a myc-tagged protein or GFP expression (Figure 3). Under normal culture conditions, neither transgene product was detected (Figures 3A and 3C). However, several hours following a 30 min heat shock at 34°C, expression is clearly evident (Figures 3B and 3D). This confirms that the HSP70 promoter is not significantly leaky at 25°C and will generate strong and ubiquitous expression following heat shock at 34°C.

Transgenic F0 animals were generated, which carry integrated double transgenes comprising active forms of either the BMP or Notch receptor under the control of the HSP70 promoter, along with a marker that expresses GFP under the control of the γ-*crystallin* promoter (Figure 3G). Transgenics, which are identified by virtue of GFP expression in the lens of the eye, should therefore also carry the inducible transgene. When subjected to heat shock, these tadpoles will activate the pathway of interest in all cells, including those at the site of the lesioned tail. In order to validate this system functionally, transgenic animals carrying the *Noggin* transgene were subjected to heat shock at stage 14. Transgenics exhibited several defects consistent with inhibiting BMP signaling postgastrulation, such as lack of a heart and tail bud-derived somites, reduced eye size, and an abnormal gut compared to nontransgenics (Figures 3E and 3F).

The transgenic procedure of Amaya and Kroll (1999) generates a mixture of transgenic and nontransgenic F0 embryos. Nontransgenic tadpoles were used as controls for each experiment (designated WT controls), eliminating the possibility that the transgenic procedure itself can influence regenerative ability. To investigate the effect of reactivating embryonic gene expression on regenerative capacity during the refractory period, tadpoles were heat-shocked 3–4 hr before extirpation of the posterior 50% of the tail at stage 46. A further heat shock was administered each day for 3–4 days following amputation. After 7 days, the extent of regeneration was scored and the results are shown in Table 1A and Figure 4. While very few control tadpoles regenerated a tail (Figure 4A), tadpoles carrying either the active Notch or BMP pathway transgenes regenerated in most cases (Figures 4B–4D).

Expression of the constitutively active BMP receptor Alk3 frequently resulted in the formation of faithful regenerates (90% of cases; Figure 4B), perfectly aligned with the stump tissues and containing muscle, spinal

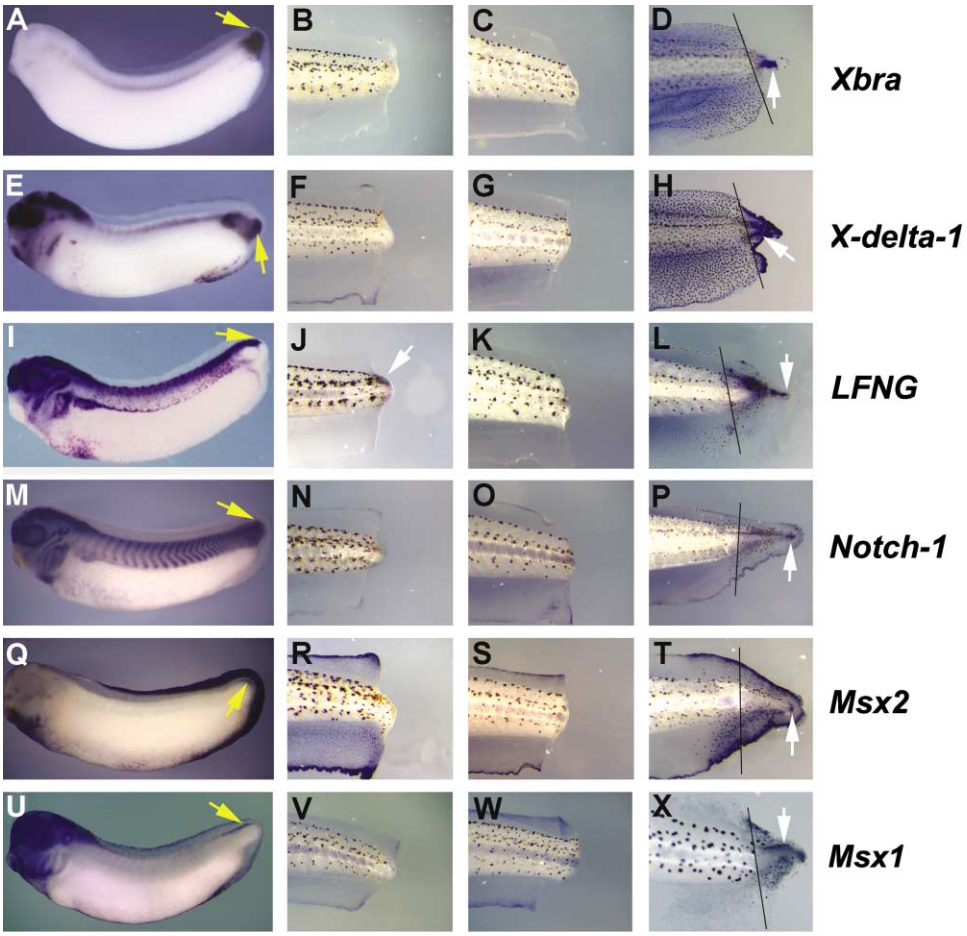

Figure 2. Analysis of Developmental Gene Expression in Nonregenerating and Regenerating Tail Tissues

In situ hybridization of genes involved in tail development.

(A, E, I, M, Q, and U) Stage 30 embryos.

(B, F, J, N, R, and V) Nonregenerating tail stumps, 50% amputated at stage 47 and fixed 3 days afterward.

(C, G, K, O, S, and W) Stage 49 tail stumps, 50% amputated and fixed immediately.

(D, H, L, P, T, and X) Regenerating tail stumps, 50% amputated at stage 49 and fixed 3 days later. Amputation level is shown by the black line.

(A–D) *Xbra* is expressed in the developing tail bud (yellow arrow) and reexpressed in the tip of regenerating tails (white arrow).

(E–H) *X-delta-1* is expressed in the posterior wall of the tail bud (yellow arrow) and fin and is reexpressed in the fin and bud region (white arrow) of regenerating tails.

(I–L) *lfng* is expressed in the dorsal tail bud (yellow arrow) and is expressed weakly at the cut surface of the spinal cord in nonregenerating tail stumps and more strongly in dorsal components of the regenerating tail (white arrows).

(M–P) *X-Notch-1* is expressed in somites, fin margin, and throughout the developing tail bud (yellow arrow) and is reexpressed in the blastema (white arrow), presomitic mesoderm, and fin margin.

(Q–T) *Msx2* is expressed in the tail bud leading edge (yellow arrow) and reexpressed throughout the blastema (white arrow) and fin margin.

(U–X) *Msx1* is expressed in the dorsal tail bud (yellow arrow) and reexpressed dorsally in the regenerating blastema (white arrow). All are lateral views, oriented with anterior to the left.

cord, notochord, and fin (Figures 4I–4K and 4O). Expression of the constitutively active Notch intracellular domain (NICD) during early regeneration results in the formation of imperfect regenerates often growing at an angle to the stump axis (73% of cases; Figure 4C). On closer examination, these regenerates were found to contain notochord and spinal cord, but muscle cells were either very few in number or totally absent (Figures 4L, 4M, and 4P). As such, NICD regenerates scored only 5 points (see Experimental Procedures) for partial regeneration (Table 1A). Mortality rate for this transgene was around 50% following heat shocks, whereas with the other transgenes used in this study, mortality was no different from wild-type siblings.

A Hyperactivated Form of Msx1 Can Induce Tail Regeneration

The BMP signaling pathway activates several known downstream targets, one of which is the homeobox transcription factor Msx1. Expression of *Msx1* is directly activated by BMP signaling (Suzuki et al., 1997), and

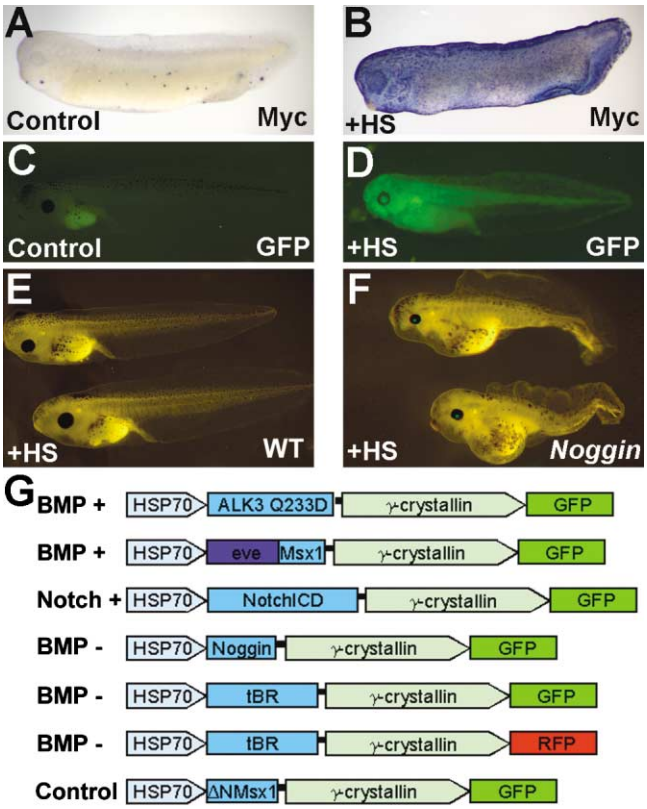

Figure 3. Properties of the Type of Constructs Used in This Study

(A and B) Embryos transgenic for a myc-tagged protein under the control of the HSP70 promoter were either heat-shocked at stage 14 (B) or not (A), and the expression was visualized by immunostaining (dark blue).

(C and D) Tadpoles transgenic for GFP under the control of the HSP70 promoter were heat-shocked (D) or not (C), and expression was viewed by fluorescence.

(E and F) Embryos heat-shocked at stage 14 develop normally in the absence of the *HSP70-Noggin-γCrys-GFP* transgene, whereas their transgenic siblings develop multiple defects consistent with BMP inhibition. Note the GFP expression in the lenses of the eyes in transgenics.

(G) Transgene constructs used in this study.

Msx1 can induce the dedifferentiation of mouse myotubes in culture from quiescent multinucleate cells to mononucleate, proliferating cells (Odelberg et al., 2000). Our results show that *Msx1* is actively transcribed in regenerating but not nonregenerating tails (Figures 2V and 2X). Furthermore, we find that *Msx1* is reexpressed in Alk3 transgenics operated during the refractory period (Figure 4F). *Msx1* is not reexpressed in WT controls or in the NICD transgenics, which form hypomorphic regenerates lacking myotomes (Figures 4G and 4H).

Msx1 is a transcriptional repressor, and replacement of the repression domain of the *Xenopus* protein with the potent repression domain from *Drosophila* evenskipped creates a hyperactive form of the protein, eveMsx1 (Yamamoto et al., 2000). In order to find out whether this reagent can mediate all effects of BMP signaling on regeneration, we made transgenic tadpoles expressing *eveMsx1* under the HSP70 promoter. These develop normally at 25°C, but following heat shock, the tails of stage 47 tadpoles, which would not normally regenerate following amputation, regenerate a well-patterned tail including myotomes, spinal cord, and notochord (83%; see Figure 4D; Table 1A). Activating Msx1 can therefore substitute for activating the BMP pathway in tail regeneration, suggesting that it functions as a key component of the regeneration mechanism. As a control, transgenic tadpoles containing a truncated version of Msx1 lacking the N-terminal repression domain were tested for stimulation of regeneration in response to heat shock. These did not regenerate any tissue following amputation of the tail at stage 47 (0%; see Figure 4E; Table 1A).

Tail Regeneration Can Be Blocked by Preventing Activation of the BMP or Notch Signaling Pathways

The above results show that activation of BMP or Notch signaling is sufficient to promote regeneration in the early *Xenopus* tadpole tail during its refractory stages. In order to show that activation of the BMP pathway is necessary for normal regeneration to occur in later stages, we generated transgenic tadpoles carrying inhibitory transgenes, expressing either the dominant-negative BMP receptor tBr (Suzuki et al., 1994) or the BMP antagonist noggin (Smith and Harland, 1992) under the control of the HSP70 promoter, again on the same construct with the γ-*crystallin-GFP* reporter (Figure 3G). Tadpoles were left to develop to stages 50–52, along with their nontransgenic siblings, and both groups were subjected to heat shock 3 hr before removal of the posterior 50% of the tail. In order to maintain transgene activity, a further heat shock was given every day during the next 6 days before scoring on day 7, and results are shown in Figure 5 and Table 1B. Among the nontransgenic group, the vast majority regenerated a full tail within 7 days (Figure 5A). Around 50% of the transgenics failed to initiate regeneration (Figures 5B and 5C), whereas the rest regenerated anything from a small spike to a full tail (Table 1B). Although the percentage regenerating is reduced to only about half that of control, the size and quality of the regenerates was markedly reduced as indicated by the reduction in the regeneration score (see Experimental Procedures) by 60%–70%. The results were similar for tBr, which is a cell surface receptor and should therefore be cell autonomous, and

Table 1. Effect of Enhancers and Inhibitors of BMP and Notch Signaling on Tail Regeneration

A

Transgene	Regeneration None	Partial	Total	N	% of Cases Regenerating	Mean Regeneration Score (of 10)	χ^2	p Value
HSP70-Alk3-γCrys-GFP	3	18	11	32	90	6.3	35.6	<0.001
WT control	46	14	3	63	27	1.6		
HSP70-eveMsx1-γCrys-GFP	4	10	9	23	83	7.3	28.3	<0.001
WT control	36	5	2	43	16	1.0		
HSP70-ΔNMsx1-γCrys-GFP	28	0	0	28	0	0		
WT control	53	0	0	53	0	0		
HSP70-NICD-γCrys-GFP	14	37	0	51	73	3.6	44.9	<0.001
WT control	39	0	0	39	0	0		

B

Transgene/Treatment	Regeneration None	Partial	Total	N	% of Cases Regenerating	Mean Regeneration Score (of 10)	χ^2	p Value
HSP70-Noggin-γCrys-GFP	10	9	2	21	52	3.1	50	<0.001
WT control	1	0	41	42	98	9.8		
HSP70-Noggin-γCrys-GFP F1	22	0	0	22	0	0		
WT control F1	0	0	15	15	100	10		
HSP70-tBR-γCrys-GFP	13	15	4	32	59	3.6	33.8	<0.001
WT control	0	1	19	30	95	9.8		
10 μM MG132	39	3	4	46	15	1.2	78.8	<0.001
DMSO control	1	1	43	45	96	9.6		

C

HSP70-Alk3-γCrys-GFP	MG132 10 μM	Regeneration None	Partial	Total	N	% of Cases Regenerating	Mean Regeneration Score (of 10)	χ^2	p Value
+	−	20	14	24	58	66	5.3		
−	−	73	16	9	97	25	1.6		
+	+	29	11	6	46	35	2.5	0.4	p > 0.1
−	+	41	14	6	61	32	2.1		

D

Transgenes	Regeneration None	Partial	Total	N	% of Cases Regenerating	Mean Regeneration Score (of 10)	χ^2	p Value
HSP70-tBr-γCrys-RFP + *HSP70-NICD-γCrys-GFP*	1	3	1	5	80	5	13.4	<0.001
WT controls	14	0	0	14	100	10		

(A) Induction of regeneration during refractory period.
(B) Inhibition of regeneration in older tadpoles.
(C) Epistasis: effect of blocking Notch signaling on BMP-induced regeneration during the refractory period.
(D) Epistasis: effect of induced Notch activity on regeneration in older tadpoles with blocked BMP signaling.

for noggin, which is a secreted factor and is likely to act nonautonomously.

Notch signaling during regeneration was inhibited using the cell-permeable protease inhibitor MG132, which blocks the γ-secretase proteolysis required for generation of the Notch intracellular domain (NICD; De Strooper et al., 1999). We have shown previously that this compound inhibits the neurogenic and tail-forming roles of Notch in *Xenopus* embryos (Beck and Slack, 2002). MG132 blocks tail regeneration very effectively without affecting the overall growth of the tadpole (Table 1B; Figure 5D).

Site of Transgene Insertion Influences Regeneration Phenotype in F0 Animals

In F0 transgenics, every individual has a different insertion site and copy number, so the level of transgene expression between individuals can be very variable. We were interested to know whether the incompleteness of the inhibition seen with BMP-inhibiting transgenes resulted from this variability. In order to test this, an F1 generation was produced by outcrossing an 8-month-old male transgenic frog harboring a single insertion site of the *HSP70-Noggin-γCrys-GFP* transgene. As expected, around 50% of the F1 individuals inherited the transgene, as seen by the presence of green fluorescent eyes. These tadpoles developed normally relative to their nontransgenic siblings and regenerated tails following amputation. However, when subjected to daily heat shocks, 22/22 failed to regenerate their tails (Table 1B; Figure 5E). No *Msx1* expression was seen in the tail stump after 2 days of regeneration (Figure 5F). Wild-type siblings all regenerated a tail (Figure 5G) and expressed *Msx1* in the distal portion of the regenerate (Figure 5H).

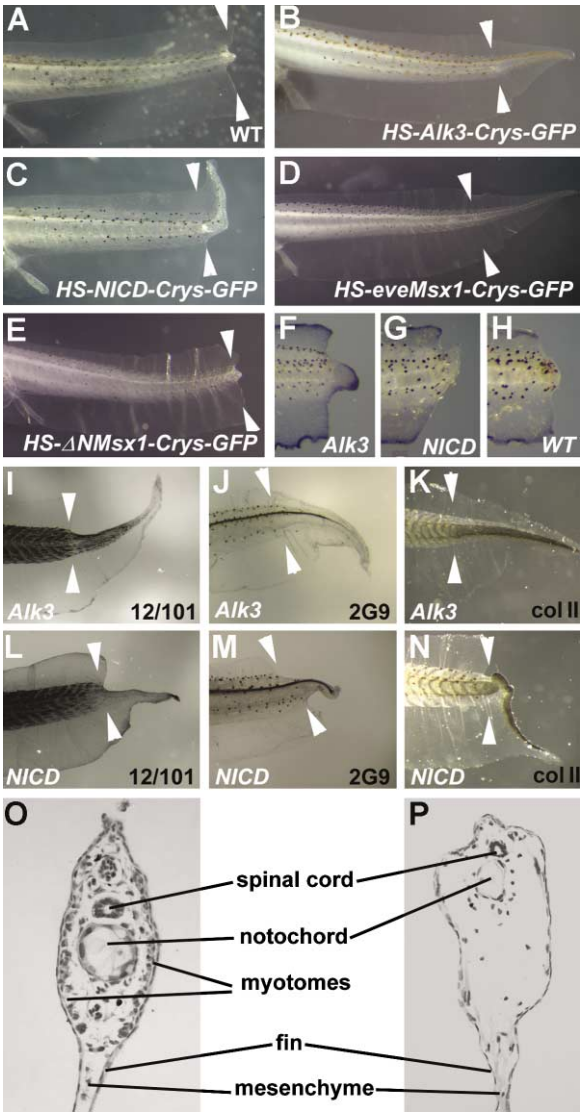

Figure 4. Activation of BMP or Notch Signaling Pathways during the Refractory Stages Promotes Regeneration of Tail Tissues

Nontransgenic (A) and transgenic tadpole tails (C–E) shown 7 days after removal of 50% of the tail at stage 47. All tadpoles received a heat shock 3–4 hr before amputation and subsequent daily heat shocks.

(A) Wild-type (WT) tadpoles as controls for transgenics. No regeneration of tail tissues from the stump has occurred.

(B) Tadpoles transgenic for the *HSP70-Alk3-γCrys-GFP* construct can regenerate their tails completely.

(C) Tadpoles transgenic for the *HSP70-NICD-γCrys-GFP* construct partially regenerate their tails, reforming the spinal cord and notochord.

(D) Tadpoles transgenic for the *HSP70-eveMsx1-γCrys-GFP* construct can regenerate their tails completely.

(E) Replacing *eveMsx1* with the nonfunctional, N-terminal-deleted *ΔNMsx1* in the transgene cassette abolishes regeneration ability. White arrows show the level of amputation. Tails are shown in lateral view, anterior to the left and dorsal uppermost.

(F–H) Analysis of *Msx1* expression (blue staining) in refractory stage tadpoles 2 days after amputation and heat shock.

(F) *Msx1* is expressed in *HSP70-Alk3-γCrys-GFP* transgenics.

(G) *Msx1* is not expressed in *HSP70-NICD-γCrys-GFP* transgenics.

(H) *Msx1* is not expressed in nontransgenic sibling controls.

(I–N) Analysis of tissue composition of 7 day regenerates during the normal refractory period. Immunohistochemical staining (black/brown) for muscle (12/101), spinal cord (2G9), and notochord (collagen II) in transgenic tadpoles subjected to tail amputation.

(I–K) Tadpoles expressing *HSP70-Alk3-γCrys-GFP* regenerate muscle (I), spinal cord (J), and notochord (K).

(L–N) Tadpoles expressing *HSP70-NICD-γCrys-GFP* do not regenerate muscle (L), but do regenerate new spinal cord (M) and notochord (N).

(O and P) Transverse histological sections. Tadpoles expressing *HSP70-Alk3-γCrys-GFP* regenerate spinal cord, myotomes, notochord, and fin (O). Tadpoles expressing *HSP70-NICD-γCrys-GFP* regenerate spinal cord, notochord, and fin, but not muscle (P).

The high reproducibility of this experiment suggests that much, if not all, of the variation seen in the F0 experiments is due to position and arrangement of the transgene within the genome.

Notch Acts Downstream of BMP Signaling during Tail Regeneration

We have shown that either Notch or BMP pathways can initiate tail regeneration at early stages, and inhibition of either pathway can inhibit regeneration at later stages. The two systems may form part of a linear pathway, or act independently in parallel. If they act in a linear pathway, BMP is likely to be epistatic to Notch, because it causes regeneration of all the same tissues as Notch but also causes additional regeneration of muscle. To investigate this possibility, we carried out two types of epistasis experiment. First, we looked at the effect of removing the tails of early stage tadpoles transgenic for *HSP70-Alk3-γCrys-GFP* in the presence or absence of 10 μM protease inhibitor MG132. *Alk3* transcription, when activated by heat shock, leads to constitutive activation of the BMP pathway and promotes regeneration in early tadpoles, which would not normally regenerate. However, in the presence of 10 μM MG132, the tadpoles behave like their nontransgenic control siblings, suggesting that interfering with Notch cleavage suppresses the regenerative ability of Alk3 (Table 1C; Figures 6A–6D). A χ^2 test comparing the mean regeneration scores showed that there was no significant difference between transgenic and wild-type animals in the presence of MG132, confirming our interpretation.

Second, we also looked at the effect of blocking BMP signaling at late, regenerating, stages in the presence of an active Notch signal. This was done using double transgenics. An RFP-tagged version of *HSP70-tBr-γCrys-GFP* was made and used together with *HSP70-NICD-γCrys-GFP* to make transgenics. Previous observations by ourselves and others suggest that two transgenes will be cointegrated around 95% of the time if they are linearized with the same restriction endonuclease. Double transgenics can be identified by virtue of eye color, as they will fluoresce amber when viewed with a GFP filter due to the presence of both GFP and RFP in the lens. Tadpoles were heat-shocked 3 hr before amputating the posterior 50% of their tails, and subsequently heat-shocked every day for 7 days. Unfortunately, the mortality of the double transgenics was very high, and despite using 55 stage 49+ tadpoles from five separate transgenic experiments, only five survived the regeneration period and could be scored. However, these few cases do show a clear difference of behavior when compared to both the WT control tadpole group

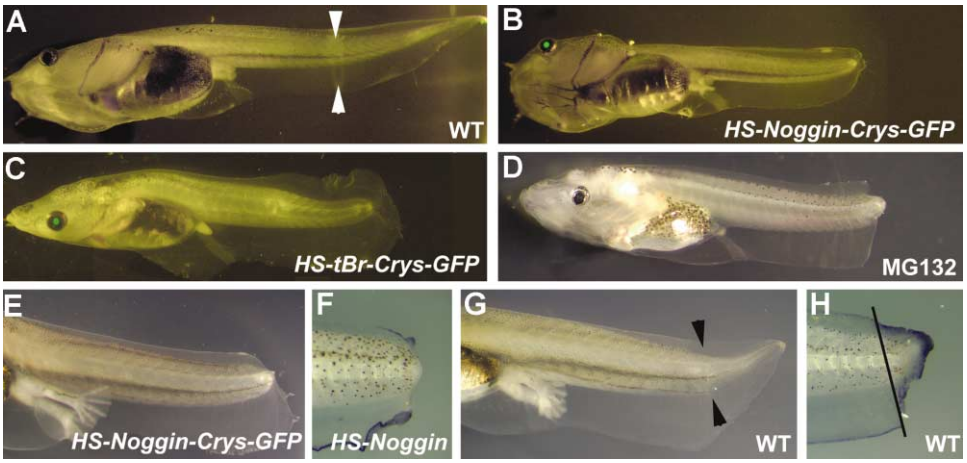

Figure 5. Inhibition of the BMP or Notch Signaling Pathways Prevents Tail Regeneration

Nontransgenic (A and D) and transgenic F0 tadpoles (B and C) shown 7 days after removal of the posterior 50% of the tail at stage 50/52. (A)–(C) are viewed with both fluorescent and low incident light such that the GFP in the lens can be detected, indicating the presence of the transgene. The tadpoles received a heat shock 3–4 hr before amputation and subsequent daily heat shocks.

(A) A wild-type (WT) tadpole can regenerate a complete tail in 7 days. White arrowheads indicate the level of amputation.

(B) Transgenic tadpole carrying the *HSP70-Noggin-γCrys-GFP* construct. Tail regeneration was completely blocked.

(C) Transgenic tadpole carrying the *HSP70-tBr-γCrys-GFP* construct. Tail regeneration was completely blocked.

(D) Stage 49 WT tadpole cultured in 10 μM MG132 immediately after removal of the posterior half of the tail, for 7 days. Tail regeneration was completely blocked.

(E–H) Regeneration phenotype is linked to inheritance of the transgene in F1 animals derived from crossing a male individual carrying the *HSP70-Noggin-γCrys-GFP* to a wild-type female. Following heat shocks and amputation, transgenic animals failed to regenerate their tails (E) and *Msx1* (blue staining) was not expressed in the stump (F). WT siblings regenerated normally (G), and expressed *Msx1* in the blastema (H). Black arrowheads in (G) and a black line in (H) mark the level of amputation.

or to tadpoles containing only the *tBr* transgene. The regenerates are partial, consisting of spinal cord and notochord without muscle, and resemble the Notch-induced regenerates in early refractory stage tadpoles (Table 1D; Figures 6E and 6F). This suggests that the situation is very similar to that in embryonic tail development, where we have shown that Notch acts downstream of BMP in formation of the caudal neural tube but that Notch is not required for muscle formation (Beck et al., 2001). Taken together, the current epistasis results are consistent with a model where BMP signaling acts upstream of Notch in spinal cord regeneration but that regeneration of muscle requires BMP and not Notch signaling.

Discussion

Stage Dependency of Axial Regeneration Capacity in a Vertebrate

While mammalian muscle, bone, and skin can regenerate to some extent via stem cell expansion (Stocum, 2001), the regeneration of complete appendages, tails, or damaged spinal cord does not take place in mammals. In some other animals these do occur via epimorphic regeneration, examples of which can be found scattered throughout the metazoan phyla. The widespread but patchy occurrence of epimorphic regeneration capacity suggests that this is an ancestral trait that has been generally selected against and therefore lost repeatedly during evolution (Goss, 1991; Korscheldt, 1927). If this interpretation is correct, it is likely that the ability to regrow a limb or repair a damaged spinal cord remains dormant in all vertebrates. Tail regeneration of vertebrates occurs in lizards, urodeles, and some fish (Bernhard and Wagner, 1992; Bryant and Bellairs, 1970; Geraudie and Singer, 1992; Iten and Bryant, 1976; Santamaria and Becerra, 1991; Simpson, 1970; Spallanzani, 1769), and has been most extensively studied in urodeles. The urodele's extensive regenerative capability results from ability of stump tissue underlying the wound epidermis to dedifferentiate and reenter the cell cycle (Brockes and Kumar, 2002; Echeverri et al., 2001; Lo et al., 1993; Tanaka et al., 1997, 1999). Our results show for the first time, to our knowledge, that axial regenerative ability in an anuran amphibian is not continuous throughout development. *Xenopus* can regenerate axial tissue at stage 42, but from stages 45 to 47, as in higher vertebrates, the wound heals over with "normal" epidermis, preventing the progression of the regenerative process. As the tadpole progresses, axial regeneration ability is reactivated with the formation of a wound epithelium, mobilization of cells to form a regeneration bud, and finally, replacement of the original tissues. The behavior of the tail can be contrasted with the behavior of the limbs in *Xenopus*, which can regenerate during the early tadpole stages before ossification of the limb skeleton, but lose regenerative capacity as differentiation becomes completed (Dent, 1962; Muneoka et al., 1986; Overton, 1963; Wolfe et al., 2000).

Generation and Regeneration of the Axial Tissues Use the Same Genetic Pathways

Notwithstanding the fact that capacity to regenerate the tail is reacquired postembryonically, key to the process

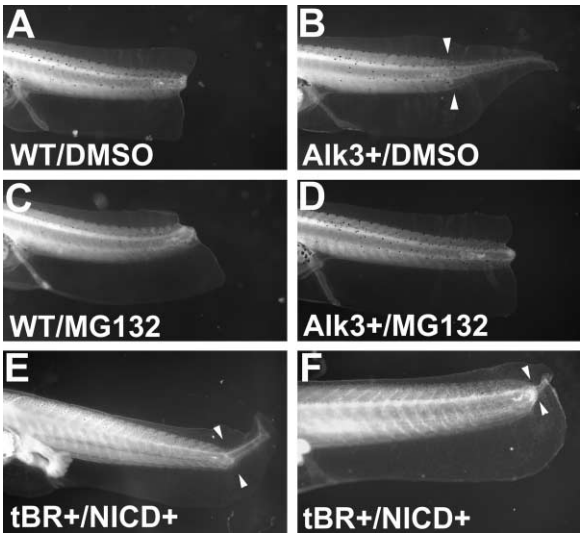

Figure 6. Epistasis Experiments Suggest that BMP Acts Upstream of Notch in Tail Regeneration

(A–D) Effect of a Notch inhibitor on BMP pathway-driven refractory stage regeneration. Tadpoles were amputated at stage 47 and given daily heat shocks for 1 week.

(A) Wild-type (WT) tadpoles do not regenerate at this stage.

(B) Sibling tadpoles expressing the *HSP70-Alk3-γCrys-GFP* regenerate all the tail tissues.

(C and D) Neither WT (C) nor transgenic tadpoles (D) will regenerate any tail tissue following treatment with 10 μM Notch proteolysis inhibitor MG132.

(E and F) Effect of combined inhibition of BMP signaling and activation of the Notch pathway. The tadpoles express both *HSP70-tBR-γCrys-GFP* and *HSP70-NICD-γCrys-RFP*. Following amputation after stage 50, when WT animals regenerate tails normally, these double transgenics regenerate only notochord and spinal cord tissue. No new muscle is formed. White arrowheads mark the site of amputation in (B, E, and F).

of regeneration is the reactivation of genetic pathways utilized during development. We have shown previously that BMP signaling is required for formation of all tail axial tissues during development (Beck et al., 2001). Here we show that components of this pathway are also specifically reexpressed during the early stages of regeneration and that BMP signaling is both sufficient and necessary for tail regeneration in this vertebrate model organism. We have shown that the *Msx1* gene is likely to be the main target of BMP signaling in this case. In limb development and regeneration it has been suggested that Msx transcription factors maintain the proliferating and undifferentiated state of the cells in the blastema region (Carlson et al., 1998; Koshiba et al., 1998). *Msx* expression is downregulated as limb regeneration progresses, and ectopic expression is also known to transform mouse myotubes into mononucleate cells capable of proliferation (Odelberg et al., 2000) and to inhibit redifferentiation in cultured myoblasts (Song et al., 1992). *Msx* expression also correlates with ear punch healing in the MRL (healer) mouse (Heber-Katz, 1999) and digit tip regeneration in wild-type mice (Reginelli et al., 1995). Taken together, this work suggests real potential for provoking regenerative behavior by manipulation of the BMP pathway and its targets.

In addition to demonstrating the role of BMP signaling, we also present evidence for the role of a second pathway, active specifically in spinal cord and notochord regeneration. The Notch signaling pathway, among its many other roles, drives formation of the tail spinal cord during embryogenesis (Beck and Slack, 1999, 2002). Here we show that Notch and its ligand, Delta, are specifically reactivated in regenerating tails and that the Notch pathway modulator, lunatic fringe, is strongly upregulated. The addition of an inhibitor known to suppress Notch cleavage and subsequent transduction of the signal will prevent tail regeneration, and activation of the Notch pathway during the early nonregenerating stage is sufficient to induce regeneration of spinal cord, notochord, and some fin, but muscle cells are either found rarely, or not at all.

We have previously shown that, in development, BMP signaling acts in two ways to stimulate tail growth, first to activate the Notch pathway, necessary for generation of the neural tube, and second, by a Notch-independent mechanism to generate segmented muscle. In our epistasis experiments, a Notch inhibitor can prevent regeneration induced by ectopic BMP signaling and a constitutive form of Notch can provoke spinal cord and notochord regeneration in the absence of BMP signaling. This suggests that a very similar relationship between the two pathways exists in regeneration and in embryonic development. Furthermore, activation of BMP but not Notch induces expression of *Msx1* in the regenerating tissue, suggesting that Msx1 is a specific target of BMP signaling during regeneration.

Experimental Procedures

Transgene Constructs

We designed a modified form of the pBSIIKS+ plasmid vector (Stratagene) to enable the design and insertion of double transgenes into the genome of *Xenopus*, called *HGEM* (*h*eat shock *g*reen-eyed *m*onster). This contains a *Xenopus HSP70* promoter (Marsh-Armstrong et al., 1999) upstream of a multicloning site (MCS), followed by a 6× myc tag and SV40 poly A, and the *Xenopus* γ-*crystallin* promoter driving expression of GFP (kind gift of Rob Grainger's lab) as a visible marker for transgene insertion. The *HGEM* vector was used to make several transgene constructs used in this study (see Figure 3G) by inserting the gene to be expressed into the MCS.

The entire *Xenopus Noggin* coding region was amplified from *CSKA-Noggin* (a kind gift of Betsy Pownall) using proofreading Pfu polymerase (Promega) and primers to amplify amino acids 1–222 (5′-CCCAAGCTTATGGATCATTCCCAGTGCCT-3′; contains an engineered HindIII site upstream of the ATG; 3′-GCATGAGCATTTG CACTC-5′). The product was digested with HindIII and cloned into the *HGEM* vector HindIII and SmaI sites to generate a myc fusion at the 3′ end of Noggin. *HSP70-Noggin-γCrys-GFP* was linearized with NotI before use in transgenics. Dominant-negative *Xenopus* truncated BMP receptor (tBR) from *pSP64T- tBR* (Suzuki et al., 1994) was excised using HindIII and SmaI to include the globin 5′ and 3′ regions, and cloned into the HindIII and StuI sites of HGEM, eliminating the myc tag. *HSP70-tBr-γCrys-GFP* was linearized with NotI before use in transgenics. An RFP version of this construct was also made by replacing GFP with RFP (Clontech) to make *HSP70-tBr-γCrys-RFP*.

Alk3 (Hsu et al., 1998), a constitutively active form of the BMPR-1 receptor (Gln 233-Asp; a kind gift of Richard Harland's lab), was excised with EcoRI, XbaI (blunt filled) into EcoRI, and SmaI of *HGEM*. *HSP70-Alk3-γCrys-GFP* was linearized with NotI before use in transgenics. A truncated, inactive form of *Xenopus Msx1* lacking the repression domain (Δ*NMsx1* in *CS2*+; Yamamoto et al., 2000) was excised with HindIII and EcoRV and cloned into the HindIII and StuI

sites of *HGEM* to make *HSP70-ΔNMsx1-γCrys-GFP*. Hyperactive *Msx1* (*eveMsx1* in *CS2+*; Yamamoto et al., 2000) was cloned using the same sites to make *HSP70- eveMsx1-γCrys-GFP*. To activate the Notch pathway, a constitutively active form of the *Xenopus Notch-1* intracellular domain in *CS2+* (*NICD*; Coffman et al., 1993) was used. The coding region was excised with EcoRI and XbaI (filled with klenow) and cloned into *HGEM* using the EcoRI and StuI sites. *HSP70-NICD-γCrys-GFP* was linearized with NotI before use in transgenics.

Histology, Marker Expression, and Immunohistochemistry

Transverse paraffin sections through regenerating transgenic tails were stained using borax carmine and picroblueblack as previously described (Godsave and Slack, 1988). Whole-mount in situ hybridization for the tail bud markers *Xbra, X-delta-1, X-Notch-1, lunatic fringe, BMP-4*, and *Msx1* has been described previously (Beck and Slack, 1998; Beck et al., 2001; Christen and Slack, 1998). An *Msx2* EST was obtained from the UK HGMP Resource Centre. Antisense DIG-labeled *Msx2* probe was made from Image 3199584 EST cut with ClaI and transcribed with T7 RNA polymerase. For whole-mount immunohistochemistry, tadpoles were fixed in Dent's fixative (80% MeOH, 20% DMSO) for 90 min at 4°C and stored in MeOH at −20°C. Monoclonal 12/101 antibody was used for the detection of muscle (Kintner and Brockes, 1984), the anti-neural monoclonal antibody 2G9 (Jones and Woodland, 1989) was used for the detection of spinal cord, and anti-collagen II (ICN; 1/600) was used for the detection of notochord. Anti-mouse-POD IgG (Sigma) was used as secondary antibody in all cases (1/1000), and the signal was developed using an enhanced DAB staining kit (Amersham). Myc staining was detected using anti-Myc antibody (Evan et al., 1985) and detected by incubation with anti-mouse-AP IgG (Vector labs) followed by color development using BM purple precipitating solution (Roche).

Transgenic *Xenopus* Tadpoles

Transgenic *Xenopus laevis* tadpoles were made according to the method of Amaya and Kroll (1999) except that the final high speed cytoplasmic egg extract was heated to 80°C for 8 min before brief centrifugation at 70k rpm, and 10 μl of the resulting supernatant was used per reaction. Restriction enzyme was omitted from the reaction, and sperm nuclei were frozen in aliquots in sperm suspension buffer before use.

Activation of the HSP70 Promoter

Tadpoles were grown at 25°C in 10% isotonic NAM up to stage 48 and then transferred to a recirculating aquarium. For activation of the transgene heat shock promoter, tadpoles were placed in 100 ml jars containing warmed water at 34°C, and the jars were placed in a water bath at the same temperature for 30 min.

Tail Amputations

If the amputation followed a heat shock, a 3–4 hr period at 25°C was allowed for transgene expression. Tadpoles were anaesthetized in 1/4000 MS222 (3-aminobenzoic acid ethyl ester; Sigma) made up in NAM/10 without gentamycin, and 50% of the tail was removed using iridectomy scissors (John Weiss). Amputated tadpoles were allowed to recover from anesthesia in NAM/10 without gentamycin before returning to aquarium tanks. For Notch inhibition experiments, tadpoles were incubated following recovery in petri dishes containing 10 μM MG132 (Calbiochem) in NAM/10 without gentamycin, and this was refreshed daily. MG132 was made up as a 10 mM stock in DMSO, and controls were treated with an equivalent DMSO concentration as a vehicle control.

Photography and Microscopy

GFP was visualized in live tadpoles, anaesthetized as above, using a Leica Fluo III fluorescent dissecting microscope with a GFP2 filter set. Images were captured using a SPOT RT camera (Diagnostic Instruments). For differential interference contrast microscopy (DIC), tadpoles were fixed in MEMFA for 2 hr and then rinsed with PBS before gradual transfer to 100% glycerol. Tails were mounted in glycerol under a cover slip and examined using a Nikon Eclipse E800 compound microscope with a 10× DIC objective.

Regeneration Scoring

In order to discriminate between complete and partial regeneration, we used a scale whereby 10 points are given for each tail in which regeneration is complete (with respect to controls, with muscle segmentation apparent), 5 points for a spike, indicating partial regeneration, and 0 points for tails that fail to regenerate at all. The mean regeneration score was calculated from these scores, while the percentage of regeneration was calculated by including both partial and complete regenerates as positive. Embryos made during the transgenic procedure, but which were negative for transgene expression, were retained as controls and were treated alongside transgenics in all cases. Where clear differences were seen between the mean regeneration scores, this was shown to be highly significant using χ^2 tests based on the null hypothesis that regeneration ability in experimental (transgenic) embryos is the same as in wild-type controls (Table 1).

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References

Amaya, E., and Kroll, K.L. (1999). A method for generating transgenic frog embryos. Methods Mol. Biol. *97*, 393–414.

Beck, C.W., and Slack, J.M.W. (1998). Analysis of the developing *Xenopus* tail bud reveals separate phases of gene expression during determination and outgrowth. Mech. Dev. *72*, 41–52.

Beck, C.W., and Slack, J.M.W. (1999). A developmental pathway controlling outgrowth of the *Xenopus* tail bud. Development *126*, 1611–1620.

Beck, C.W., and Slack, J.M.W. (2002). Notch is required for outgrowth of the *Xenopus* tail bud. Int. J. Dev. Biol. *46*, 255–258.

Beck, C.W., Whitman, M., and Slack, J.M.W. (2001). The role of BMP signaling in outgrowth and patterning of the *Xenopus* tail bud. Dev. Biol. *238*, 303–314.

Bernhard, Y.M., and Wagner, G.P. (1992). Regeneration in *Salaria pavo* (*Bleniidae, teleostei*). Anat. Embryol. (Berl) *186*, 153–165.

Bienz, M. (1984). Developmental control of the heat shock response in *Xenopus*. Proc. Natl. Acad. Sci. USA *81*, 3138–3142.

Bosco, L. (1979). Expression of regenerative capacity of caudal spinal cord during the larval development of *Xenopus laevis*. Acta Embryol. Exp. (Palermo) *3*, 275–286.

Brockes, J.P. (1997). Amphibian limb regeneration: rebuilding a complex structure. Science *276*, 81–87.

Brockes, J.P., and Kumar, A. (2002). Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat. Rev. Mol. Cell Biol. *3*, 566–574.

Bryant, S.V., and Bellairs, A. (1970). Development of regenerative capacity in the lizard, *Lacerta vivipara*. Am. Zool. *10*, 167–173.

Carlson, M.R., Bryant, S.V., and Gardiner, D.M. (1998). Expression of *Msx-2* during development, regeneration, and wound healing in axolotl limbs. J. Exp. Zool. *282*, 715–723.

Christen, B., and Slack, J.M.W. (1998). All limbs are not the same. Nature *395*, 230–231.

Coffman, C., Skoglund, P., Harris, W., and Kintner, C. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus* embryos. Cell *73*, 659–671.

Dent, J.N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. J. Morphol. *110*, 61–78.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., et al. (1999). A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. Nature *398*, 518–522.

Echeverri, K., Clarke, J.D., and Tanaka, E.M. (2001). In vivo imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema. Dev. Biol. *236*, 151–164.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Filoni, S., and Bosco, L. (1981). Comparative analysis of the regenerative capacity of caudal spinal cord in larvae of several anuran amphibian species. Acta Embryol.Morphol. Exp. *2*, 199–226.

Filoni, S., Bosco, L., and Carlizzi, C. (1977). La rigenerazione del midello spinale della coda di larvae di *Xenopus laevis* operate negli tardivi. Rend. Acc. Naz. Lincei *63*, 440–446.

Gardiner, D.M., Carlson, M.R., and Roy, S. (1999). Towards a functional analysis of limb regeneration. Semin. Cell Dev. Biol. *10*, 385–393.

Geraudie, J., and Singer, M. (1992). The fish fin regenerate. Monogr. Dev. Biol. *23*, 62–72.

Godsave, S.F., and Slack, J.M.W. (1988). Mesoderm-inducing factors: a small class of molecules. Development *102*, 555–566.

Goss, R.J. (1969). Principles of Regeneration (New York: Academic Press).

Goss, R.J. (1991). The natural history (and mystery) of regeneration. In A History of Regeneration Research: Milestones in the Evolution of a Science, C.E. Dinsmore, ed. (Cambridge: Cambridge University Press), pp. 7–23.

Heber-Katz, E. (1999). The regenerating mouse ear. Semin. Cell Dev. Biol. *10*, 415–419.

Hsu, D.R., Economides, A.N., Wang, X., Eimon, P.M., and Harland, R.M. (1998). The *Xenopus* dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol. Cell *1*, 673–683.

Iten, L.E., and Bryant, S.V. (1976). Stages of tail regeneration in the newt, *Notophthalamus viridescens*. J. Exp. Zool. *196*, 283–292.

Jones, E.A., and Woodland, H.R. (1989). Spatial aspects of neural induction in *Xenopus laevis*. Development *107*, 785–791.

Kintner, C.R., and Brockes, J.P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. Nature. *308*, 67–69.

Korscheldt, E. (1927). Regeneration und Transplantation (Berlin: Borntraeger).

Koshiba, K., Kuroiwa, A., Yamamoto, H., Tamura, K., and Ide, H. (1998). Expression of *Msx* genes in regenerating and developing limbs of axolotl. J. Exp. Zool. *282*, 703–714.

Lo, D.C., Allen, F., and Brockes, J.P. (1993). Reversal of muscle differentiation during urodele limb regeneration. Proc. Natl. Acad. Sci. USA *90*, 7230–7234.

Marsh-Armstrong, N., Huang, H., Berry, D.L., and Brown, D.D. (1999). Germ-line transmission of transgenes in *Xenopus laevis*. Proc. Natl. Acad. Sci. USA *96*, 14389–14393.

Muneoka, K., Holler-Dinsmore, G., and Bryant, S.V. (1986). Intrinsic control of regenerative loss in *Xenopus laevis* limbs. J. Exp. Zool. *240*, 47–54.

Odelberg, S.J., Kollhoff, A., and Keating, M.T. (2000). Dedifferentiation of mammalian myotubes induced by *msx1*. Cell *103*, 1099–1109.

Overton, J. (1963). Patterns of limb regeneration in *Xenopus laevis*. J. Exp. Zool. *154*, 153–161.

Piatt, J. (1955). Regeneration in the central nervous system of amphibia. In Regeneration in the Central Nervous System, W.F. Windle, ed. (Springfield, IL: Charles C. Thomas).

Reginelli, A.D., Wang, Y.Q., Sassoon, D., and Muneoka, K. (1995). Digit tip regeneration correlates with regions of *Msx1* (*Hox 7*) expression in fetal and newborn mice. Development *121*, 1065–1076.

Santamaria, J.A., and Becerra, J. (1991). Tail fin regeneration in teleosts: cell-extracellular matrix interaction in blastemal differentiation. J. Anat. *176*, 9–21.

Simpson, S.B.J. (1970). Studies on regeneration of the lizard's tail. Am. Zool. *9*, 157–165.

Smith, W.C., and Harland, R.M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. Cell *70*, 829–840.

Song, K., Wang, Y., and Sassoon, D. (1992). Expression of *Hox-7.1* in myoblasts inhibits terminal differentiation and induces cell transformation. Nature *360*, 477–481.

Spallanzani, L. (1769). Prodromo di un opera da imprimersi sopra la riproduzioni animali (An essay on animal reproduction) (London: T. Becket and deHondt).

Stocum, D.L. (1984). The urodele limb regeneration blastema. Determination and organization of the morphogenetic field. Differentiation *27*, 13–28.

Stocum, D.L. (2001). Stem cells in regenerative biology and medicine. Wound Repair Regen. *9*, 429–442.

Suzuki, A., Thies, R., Yamaji, N., Song, J., Wozney, J., Murakami, K., and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. Proc. Natl. Acad. Sci. USA *91*, 10255–10259.

Suzuki, A., Ueno, N., and Hemmati-Brivanlou, A. (1997). *Xenopus* msx1 mediates epidermal induction and neural inhibition by BMP4. Development *124*, 3037–3044.

Tanaka, E.M., Gann, A.A., Gates, P.B., and Brockes, J.P. (1997). Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. J. Cell Biol. *136*, 155–165.

Tanaka, E.M., Drechsel, D.N., and Brockes, J.P. (1999). Thrombin regulates S-phase re-entry by cultured newt myotubes. Curr. Biol. *9*, 792–799.

Tucker, A.S., and Slack, J.M.W. (1995). The *Xenopus laevis* tail-forming region. Development *121*, 249–262.

Wheeler, G.N., Hamilton, F.S., and Hoppler, S. (2000). Inducible gene expression in transgenic *Xenopus* embryos. Curr. Biol. *10*, 849–852.

Wolfe, A.D., Nye, H.L., and Cameron, J.A. (2000). Extent of ossification at the amputation plane is correlated with the decline of blastema formation and regeneration in *Xenopus laevis* hindlimbs. Dev. Dyn. *218*, 681–697.

Yamamoto, T.S., Takagi, C., and Ueno, N. (2000). Requirement of Xmsx-1 in the BMP-triggered ventralization of *Xenopus* embryos. Mech. Dev. *91*, 131–141.